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MCP-1 Feedback Loop between Adipocytes and Mesenchymal Stromal Cells Causes Fat Accumulation and Contributes to Osteocyte and Hematopoietic Stem Cell Rarefaction in the Bone Marrow of Diabetic Patients

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Abstract

Fat accumulates in bone marrow (BM) of diabetic patients. Here, we investigated the mechanisms and consequences of this phenomenon. BM-mesenchymal stromal cells (BM-MSCs) from type-2 diabetic (T2D) patients constitutively express adipogenic markers and robustly differentiate into adipocytes (ADPCs) upon *in-vitro* induction. Moreover, T2D-BM-ADPCs paracrinally stimulate a transcriptional adipogenic program in BM-MSCs, while inhibiting osteoblastogenesis. Antagonism of MCP-1, which is pivotally expressed in T2D-BM-ADPCs, prevented the T2D-BM-ADPC secretome from converting BM-MSCs into ADPCs. In T2D mice, systemically-administered MCP-1 receptor antagonist improved metabolic control, reduced BM fat, and restored the proportion of osteocytes over osteoclasts. It also indirectly re-established the abundance of long-term versus short-term hematopoietic stem cells. We reveal a diabetic feedback loop wherein (1) BM-MSCs are constitutively inclined to make ADPCs and (2) mature BM-ADPCs, *via* secreted MCP-1, relentlessly fuel BM-MSC determination into new fat. Pharmacological inhibition of MCP-1 signalling can contrast this vicious cycle aiding bone health and hematopoiesis in diabetes.

Introduction

Type 2 diabetes (T2D) is a major risk factor for cardiovascular disease because of its damaging impact on the micro- and macro-vasculature. Patients with T2D have twice the chance of dying from ischemic events than non-diabetic (ND) subjects (1) and those who survive suffer a markedly slower and more arduous recovery (2). This worse outcome has been attributed to the deleterious effect of T2D on angiogenesis and vasculogenesis, two intertwined reparative processes brought about by local and circulating cells (3). A defect in the mobilization of progenitor cells, such as $CD34^+$ cells, from the bone marrow (BM) represents a common feature (4) and a predictor for cardiovascular mortality in diabetic patients (5, 6). Moreover, the reduced availability and functionality of circulating vasculogenic cells may be the consequence of pathogenic processes occurring within the BM microenvironment (7).

Adiposity, typically associated with T2D, does not spare the BM. We previously reported an inverted relationship between adipocytes (ADPCs) and hematopoietic stem cells (HSCs), where T2D patients have an almost two-fold increase in ADPCs coverage with a proportional loss of hematopoietic tissue volume (8). It remains unknown whether ADPC accumulation represents a trivial phenomenon or a pathogenic determinant of the BM adverse remodelling.

Fat occupies a significant portion of the bone cavity and represents approximately 10% of the total fat mass in the body of a lean individual (9). The BM adipose tissue (BMAT) constitutes a distinct category sharing some phenotypic similarities with both white (WAT) and brown adipose tissue, but its function remains largely unknown (10). BMAT expansion can be observed in multiple pathological conditions, such as calorie restriction, radiation exposure, aging, and obesity and diabetes (8, 11-13).

BM-ADPCs originate from resident mesenchymal stromal cells (MSCs), which can differentiate into either adipocytic, osteoblastic or chondrogenic lineage (12, 14). Bone and BMAT are intimately linked as their levels are often found to be inversely correlated, with a reduced bone to BMAT ratio being considered a risk factor for bone fractures (15). Mature osteoblasts inhibit the differentiation of MSCs into adipose tissue through paracrine mechanisms. Conversely, mature BM-ADPCs influence bone mass by inhibiting the progression of BM-MSCs to osteoblasts and enhancing osteoclastogenesis (16). BMAT is also involved in the maintenance of the hematopoietic niche. In mice, BMAT expansion

through obesity, aging or *via* adipogenic cell transplantation causes a decrease in $Lin^{neg}Sca1^{pos}cKit^{pos}$ (LSK), long-term $CD34^{neg}$ (LT) and short-term $CD34^{pos}$ (ST) HSCs (12). BMAT also functions as an endocrine organ, surpassing the more-widespread WAT as a source of adiponectin (ADIPOQ) (13) and secreting an extensive number of cytokines and adipokines such as Leptin (LEP), Resistin (RETN), and Monocyte Chemoattractant Protein-1 (MCP-1 / CCL2) (17, 18).

MCP-1 is attracting growing interest for its roles in obesity and diabetes. Circulating levels of MCP-1 are elevated in obese and T2D patients (19). Furthermore, gene deletion (20) and pharmacological inhibition of MCP-1 reduce visceral MAT volume in obese mice (21, 22), suggesting a direct participation in adipogenesis. MCP-1 is also involved in the modulation of macrophages and T cells, thereby inducing "WAT inflammation" in T2D (23). At the molecular level, it has been reported that MCP-1, *via* binding to its receptor CCR2, activates the transcription of MCP-1 Induced Protein (MCPIP), which promotes adipocyte differentiation in a peroxisome proliferator-activated receptor γ (PPARγ)-independent manner (24, 25).

The present study aims to unveil the molecular mechanisms and consequences of ADPCs accumulation in BM of T2D patients. Results highlight a feedback loop mechanism by which mature T2D-BM-ADPCs release high levels of MCP-1 which stimulates the differentiation of BM-MSCs into new ADPCs while inhibiting osteogenesis. Importantly, *in-vivo* antagonism of the MCP-1/CCR2 signalling in T2D mice not only decreased BMAT accumulation but also rescued osteocyte and LT-HSC depletion.

Material and Methods

Patient Recuitment

Patients undergoing hip replacement surgery were recruited under informed consent at the Avon Orthopaedic Centre, Southmead Hospital, Bristol, UK. The study protocol complied with the Declaration of Helsinki, was covered by institutional ethical approval (REC14/SW/1083 , REC14/WA/1005) and registered as an observational clinical study in the NIHR Clinical Research Network Portfolio, UK Clinical Trials Gateway and ClinicalTrials.gov.

T2D was diagnosed according to the American Diabetes Association guidelines. Specifically, it was defined as (a) Patient/referring doctor reports a previous diagnosis of diabetes, (b) HbA1c> 48mmol/mol, (c) Off insulin for at least 12 months after diagnosis. Exclusion criteria comprised acute disease/infection, immune diseases, current or past hematological disorders or malignancy, unstable angina, recent (within six months) myocardial infarction or stroke, critical limb ischemia, liver failure, dialysis due to renal failure, pregnancy, and lack of consent to participate. Available data on patients characteristics are provided in **SOI Table 1**.

Human BM Isolation

BM samples were obtained from scooped femur heads remaining from hip replacement surgery. During the total hip replacement procedure, the femoral head was removed with a saw and the proximal femoral canal was opened with reamers and rasps. The BM displaced into the wound was scooped into a sterile pot with a curette. Superfluous cancellous bone was removed from the femoral neck and proximal metaphysis and placed with the marrow. The sample was decanted into a collection tube with 0.5M EDTA pH8. Only material which would otherwise be discarded was collected for study.

Histology

Bone samples were fixed in 1% paraformaldehyde (ThermoFischer, Loughborough, UK), decalcified with 10% formic acid and embedded in paraffin. Blocks were sectioned on a rotary microtome at 2µm and the samples were stained with H&E)sigma).

Human BM Cell Isolation and Culture

BM samples were stratified on Ficoll Histopaque 1077 (ThermoFischer) and centrifuged without acceleration or brake at 300*g* for 30 minutes at 25°C.

$1x10^7$ mononuclear cells from the Ficoll separation were were seeded in plastic flasks in αMEM basal media (ThermoFischer) supplemented with 20% foetal bovine serum (FBS, Gibco) for 48 hours at 37°C 5% CO_2. The adherent cells were considered BM-MSCs and expanded in αMEM supplemented with 20% FBS.

Cells from the floating phase were collected, strained through a 100μm filter and washed with HBSS (Sigma, Gillingham, UK). Cells put into culture using the modified ceiling culture method (26). $1x10^7$ cells were seeded in a well of a 12 well culture plate (CellStar, Cardiff, UK), which was filled with αMEM with 20% FBS and a 22mm borosilicate glass coverslip (VWR, Leighton, UK) was added on top of the well. Cells were incubated for 4 days and then "flipped" and put inside a well of a 6 well plate containing αMEM. ADPCs were incubated 48 hours in FBS-free αMEM. The conditioned media (CM) was then harvested and filtered through a 22μm filter (Sigma).

ELISA

Chemokines were quantified using ELISA kits (Biotechne, MN, USA) and following the manufacturer's protocol using 100μL of CM. Glycated and total haemoglobin was measured using ELISA kits (Generon, Slough, UK) using mouse serum diluted 1:500.

Gene Expression Analysis

RNA Extraction and Purification

RNA was purified using the Tri-Reagent (Sigma) manufacturer's protocol and re-purified using acid-phenol phase separation. RNA was quantified using a NanoDrop (ThermoFisher).

cDNA Library Generation

Complementary DNA was generated using the High-Capacity RNA-to-cDNA Kit (ThermoFisher) and 100ng of total RNA following the manufacturer's protocol. T100 Thermal Cycler (Bio-Rad) was used.

Quantitative PCR

mRNA expression was assayed using real-time quantitative PCR (qPCR). The reaction was made with PowerSybr green master mix (ThermoFisher) and 200nM gene-specific primers (**SI Table 2**), in a QuantStudio 6 Flex RT-PCR machine (ThermoFisher).

Flow Cytometry

Freshly isolated, mouse BM cells or cultured BM-MSCs were labelled with primary antibodies in staining buffer (HBSS supplemented with 1% BSA), acquired with a LSR Fortessa X20 (BD, Oxford, UK) and quantification of the antigenic profile was performed using the FlowJo v10 software (FlowJo, LLC). Antibodies used are listed in **SI Table 3**.

Differentiation of BM-MSCs

Adipose differentiation was induced by treatment with 1μM dexamethasone (Sigma), 0.5mM IBMX (Sigma), 10μg/ml Insulin (Sigma) and 1μM Indomethacin (Sigma) in DMEM supplemented with 10% FBS for 21 days, changing the media every 3 days. Cells were fixed with 10% formaldehyde (Sigma), treated with 60% isopropanol (Sigma) and exposed to Oil Red O (ORO, Sigma) working solution. Osteoblast differentiation was induced by treatment with 0.1mM dexamethasone, 50μg/ml L-ascorbic acid 2-phosphate (Sigma) and 10mM β-glycerophosphate (Sigma) in DMEM supplemented with 10% FBS for 14 days, changing the media every 3 days. Cells were fixed with Ice-cold methanol (Sigma) and treated with a solution of nitro-blue tetrazolium chloride (ThermoFisher) and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine (ThermoFisher) to assess alkaline phosphatase activity.

Animal Studies

Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (The Institute of Laboratory Animal Resources, 1996) and with the approval of the University of Bristol and the British Home Office (Licence: 30/2811). As a model of T2D and obesity, we used 4-week-old male obese leptin-receptor homozygous mutant C57BLKS/J-*Lepr*^{db}/*Lepr*^{db}/Dock7+ (*Db/Db*) mice (Charles River, Harlow, UK). Age- and sex-matched lean heterozygous C57BLKS/J -*Lepr*^{db}/*Lepr*^{WT} Dock7+ (*Wt/Db*) mice served as controls. Animals were fed standard chow (Charles River) and provided water ad libitum.

*M*ice were randomly allocated to receive 4 grams/kg/day of the MCP-1 receptor antagonist RS504393 (Tocris, Bristol, UK) diluted in DMSO in drinking water (1% of water volume) or the equivalent volume of DMSO for 8 weeks. Glucose urine was measured using Diastix urinalysis colorimetric reagent strips (Bayer, Reading, UK).

Statistical Analysis

Values are presented as mean ± SEM. Two-tailed independent samples t-test was used to compare T2D and ND groups assuming equal variances. One- and Two-Way ANOVA were used to compare four group experiments, followed by multivariate analysis to compare each group individually. P-values <0.05 were considered statistically significant.

Results

Features of fat accumulation in the BM of patients with T2D

As we have previously described, patients with T2D show a remarkable remodelling of the BM at a cellular level. H&E staining shows a large increase in the area covered by ADPCs in the BM of T2D patients over ND controls (**Figure 1 a-b**). This effect is due to both an increase in ADPC number and size (**Figure 1 c-e**).

Diabetic BM-MSCs have an increased propensity to differentiate into ADPCs

We investigated if adipogenic pre-determination of BM-MSCs was the cause and source of adipocyte accumulation in the diabetic BM. We first measured the effect of T2D on the expression of a spectrum of genes implicated in adipogenesis (**Figure 2 a**). Results show that T2D induces an increase in the mRNA levels of pro-adipogenic genes *C/EBP*α, *PDGFR*β and *IGFR1*. In addition, T2D caused the down-regulation of the anti-adipogenic genes *SIRT1* and *GLI1*. *STAT5a*, which can be pro- and anti-adipogenic, was also downregulated in T2D-BM-MSCs. FACS analysis of cultured BM-MSCs showed a 2-fold increase in PDGFRβ protein levels in the T2D group, whereas the pre-adipocyte factor PREF-1 remained unchanged (**Figure 2 b**).

Next, we examined the effect of T2D on BM-MSC differentiation into ADPCs or osteoblasts. Quantification of ORO-positive cells show an increase in the adipogenic activity of T2D-BM-MSCs. (**Figure 2 c i-ii**). Quantitative real-time PCR analysis also shows that T2D-BM-MSCs express higher transcript levels of the ADPC differentiation markers Fatty Acid Binding Protein 4 (*FABP4*), *ADIPOQ* and *PPAR*γ (**Figure 2 c iii-v**). These results indicate that T2D confers BM-MSCs with an enhanced epigenetic propensity into adipogenesis. On the other hand, T2D did not influence the osteogenic differentiation of BM-MSCs, as assessed by the alkaline phosphatase activity assay (**Figure 2 d i-ii**).

Mature BM-ADPCs modulate BM-MSC differentiation through paracrine mechanisms

We investigated the possibility that T2D adipocytes directly interfere with BM-MSC differentiation, creating an imbalance in the typical lineage specification. BM-MSCs from ND subjects were exposed to adipocyte or osteoblast differentiation media, which were supplemented with CM from cultured ND-BM-ADPCs (ND-CM) or T2D-BM-ADPCs (T2D-CM) or no CM as a control group (No CM). We

observed that the T2D-CM increases the differentiation of BM-MSCs into ORO-positive ADPCs over the levels found in groups treated with ND-CM or No CM (**Figure 3 a i-ii**). The enhanced activation of BM-MSC adipogenesis by the T2D-CM was associated with a greater induction of the *ADIPOQ* and *PPARγ* expression (**Figure 3 a iii-iv**), whereas *FABP4* was similarly upregulated among all groups (**Figure 3 a v**). These data suggest T2D causes an incremental feedback loop by which BM-ADPCs will increase MSC fate toward adipogenesis, which will, in turn, stimulate more BM-MSCs to do the same. We next examined the effect of T2D on osteoblast differentiation. Osteoblastogenesis assays indicate that the ND-CM increases the BM-MSC differentiation into alkaline phosphatase-positive osteoblasts. In contrast, this stimulatory effect was negated to the T2D-CM (**Figure 3 b i-ii)**. This pattern was confirmed when assessing the mRNA expression of the osteoblast marker *RUNX2* (**Figure 3 b iii**). Altogether these results indicate that BM-ADPCs of T2D patients promote a paracrine induction of BM-MSC adipogenesis at the expense of osteoblastogenesis.

T2D alters the BM-ADPC secretome

To identify which factor secreted by BM-ADPCs is responsible for the regulation of BM-MSCs fate determination we investigated by qPCR the mRNA expression of multiple adipokines and cytokines in freshly harvested human BM-ADPCs. We found that the adipokines, *LEP* and *RETN* were upregulated in T2D-BM-ADPCs, while *IGF1*, *PEDF* and *ADIPOQ* were downregulated (**Figure 4 a i**). Cytokine expression analysis revealed a large increase in *TNFα* and *MCP-1* levels in T2D-BM-ADPCs as well as a modest increase in metalloproteinase 2 (*MMP2*). We also observed that T2D reduced the levels of *IL18*, *MIF* and Angiopoietin 1 (*ANGPT1*) (**Figure 4 a ii)**. We next verified if the observed transcriptional changes were associated with similar modifications of secreted proteins. ELISA of the ADPC-CM confirmed that the levels of LEP and RETN were increased in T2D (**Figure 4 b i-ii**) and ADIPOQ was decreased (**Figure 4 b iii**), while PEDF protein levels were comparable between the two groups (**Figure 4 b iv**). We observed a striking increase in the secretion of MCP-1 protein by T2D-BM-ADPCs as well as a modest increase in MMP2 and IFNγ (**Figure 4 b v-vii**). TNFα was similar between the two groups (**Figure 4 b viii**) and ANGPT1 was increased in the T2D-BM-ADPC CM (**Figure 4 b ix**). Altogether, these data indicate T2D has a profound effect on the BM-ADPC secretome, with MCP-1 being the most

prominently affected factor. This latter phenomenon is well conserved among species as we have confirmed the elevated MCP-1 levels in the CM of BM-ADPCs from obese, diabetic *Db*/*Db* mice as compared with lean ND *Wt*/*Db* controls (**Supplemental Figure 1**).

MCP-1 plays a key role in T2D-ADPC-induced promotion of BM-MSC adipogenesis

MCP-1 and its receptor CCR2 have been implicated in obesity through the promotion of ADPC differentiation and consequent increase in adipose tissue mass (27, 28). However, it remains unknown if MCP-1 plays a role in fat accumulation within the BM of T2D patients. To verify this possibility, we performed adipogenesis assays on human BM-MSCs as previously, but with the addition of the MCP-1 receptor antagonist RS504393 or its vehicle. Inhibition of MCP-1 signalling moderately decreased BM-MSC adipogenesis in the No-CM and ND-CM groups (**Figure 5 a**). In the T2D-CM group, where adipogenesis was increased, MCP-1 antagonism resulted in an inhibition of adipogenesis to levels even lower than the No-CM group (**Figure 5 a-b**). These phenomena were associated with remarkable changes in the expression of ADPC markers. The antagonist reduced the *ADIPOQ* mRNA expression in BM-MSCs exposed to No-CM or ND-CM and completely abrogated the inductive action exerted by the T2D-CM (**Figure 5 c**). Secondly, RS504393 selectively inhibited the T2D-CM-induced upregulation of *PPARγ* (**Figure 5 d**). Thirdly, the antagonist downregulated *FABP4* mRNA levels in all experimental groups (**Figure 5 e**). Fourthly, the antagonist inhibited *MCPIP*, which was selectively upregulated in BM-MSCs following exposure to T2D-CM (**Figure 5 f**). This latter result confirms the effective inhibition of signalling downstream to the MCP-1/CCR2 duo.

***In-vivo* MCP-1 antagonism ameliorates the metabolic status of obese, diabetic Db/Db mice**

We posited that *in-vivo* administration of the same antagonist would result in a decrease in BM-ADPC accumulation. To validate this hypothesis, we exposed T2D Db/Db mice to either 4mg/kg/day of RS504393 (DR group) or DMSO (DD group) in drinking water for 8 weeks. We established a third group of ND Wt/Db (WT) to receive DMSO and be used as a reference group. During the 8 weeks experiment, no significant differences in weight were observed between the DD and DR groups (**SI Figure 2**). On the other hand, we observed that RS504393 treatment had an inhibitory effect on body weight (BW) gain (**Figure 6 a i**), without altering food intake (**SI Figure 2**). We found this lowered growth rate

associated with selective changes in specific fat deposits. While the weight of either cardiac or inguinal fat was similar in DD and DR groups, RS504393 treatment resulted in reduction of the epididymal fat pad weight (**Figure 6 a ii**). Histology of epididymal fat shows that ADPCs of T2D mice of the DR group exhibited smaller ADPCs on with respect to the DD group (**Figure 6 a iii-iv**). This indicates that the antagonist selectively reduces epididymal fat accumulation by acting on ADPC hypertrophy. Interestingly, we also observed RS504393 treatment ameliorated the metabolic control in T2D mice as indicated by a mitigation of glycosuria (**Figure 6 b i**) and reduction of blood glycated haemoglobin levels (**Figure 6 b ii**).

***In-Vivo* inhibition of MCP-1 reduces BM ADPC coverage and number and increases bone osteocytes in obese, T2D Db/Db mice**

After 8 weeks of treatment, when mice were 12 weeks of age, ADPC coverage at the head of the femur averaged 4% of the BM space in the ND WT group and 85% in the T2D DD group (**Figure 7 a i-ii**). RS504393 treatment blunted the effect of T2D on fat encroachment, lowering the total area covered by ADPCs from 85% to 73% (**Figure 7 a i-ii**). The decreased BM-ADPC coverage was not associated to differences in the average ADPC size (**Figure 7 a iii**), although the DR group contained more small-size ADPCs (1-10 μm^2), which are considered metabolically "healthy" compared with large ADPCs (**Figure 7 a iv**). We also observed that the antagonist caused a decrease in the number of ADPCs (**Figure 7 a v**). This decrease in number in the absence of changes in average size suggests that MCP-1 most likely affects the differentiation of BM cells into ADPCs *in-situ*.

Analysis of murine *Mcp-1* mRNA levels in whole BM did not show any difference between the 3 groups (**Figure 7 b i**). On the other hand, *Mcpip* mRNA levels was increased in the DD group as compared with the WT group with this increase being inhibited by RS504393 in the DR group (**Figure 7 b ii**). Serum and cell-free BM lavage concentrations of immunoreactive MCP-1 were elevated in T2D DD mice compared with WT controls with no differences observed between the DD and DR groups (**Figure 7 b iii-iv**),

The bone wall thickness of the tibia was reduced by 50% in T2D mice regardless of treatment (**Figure 7 c i-ii**). The density of osteocytes embedded inside the bone was also reduced the DD group compared

to the WT group (**Figure 7 c iii**). Interestingly, treatment with RS504393 significantly increased osteocyte density to 72% of the WT group; a 1.5 fold increase over the DD group (**Figure 7 c iii**). Quantification of osteoclasts indicates an increase in the T2D DD group over the levels observed in ND WT, with this effect being inhibited by RS504393 treatment (**Figure 7 c iv**). Altogether, these data indicate a remarkable reduction of adipogenesis and fortification of the osteocyte content following inhibition of the MCP-1 signalling.

Inhibition MCP-1 alleviates the consequences of T2D on HSC abundance and fate

HSCs are significantly affected both in terms of amount and functionality by T2D (8, 29), a result that was confirmed in the present investigation. We further analyzed by flow cytometry whether RS504393 could affect HSCs availability in the BM of T2D mice. We defined BM-HSCs as lineage negative (lin^{neg}) $cKit^{pos}$ and $Sca\text{-}1^{pos}$ cells. LT-HSCs were characterized as $Lin^{neg}CKit^{pos}Sca\text{-}1^{pos}CD34^{neg}$ while ST-HSCs as $Lin^{neg}CKit^{pos}Sca\text{-}1^{pos}CD34^{pos}$ (**Figure 8 a i**). The percentage of HSCs in the BM of the T2D mice was lower than that of the ND WT group (**Figure 8 a ii**) with no difference between the DD and DR groups. When considering HSC subpopulations according to their maturity, we found the T2D reduced the relative abundance of LT-HSCs (**Figure 8 a iii**) while increasing the abundance of ST-HSCs (**Figure 8 a iv**). This unbalance was corrected by treatment with the MCP-1 antagonist in the T2D DR group (**Figure 8 a iii-iv**), suggesting that T2D impinges upon the HSC fate determination and that MCP-1 inhibition blocks this effect. To verify if MCP-1 acts directly on HSCs, we exposed BM mononuclear cells to the CM of ND-BM-ADPCs or T2D-BM-ADPCs for 96 hours and analyzed the antigenic phenotypes of the HSC population by flow cytometry (**Figure 8 b i**). Treatment with BM-ADPC CM resulted in a slight but significant decrease in LT-HSCs in both ND and T2D (**Figure 8 b ii**), which was associated with a corresponding increase in ST-HSC (**Figure 8 b iii**). Co-treatment with RS504393 did not affect LT- and SC-HSC levels compared with the vehicle (**Figure 8b ii-iii**).

Discussion

The pathophysiological role of BM-ADPCs has been extensively speculated on and the consensus is that BMAT accumulation is deleterious to the integrity of the bone and hematopoietic niche (30-33). In this study, we provide novel extensive evidence about the cellular and molecular mechanisms underpinning fat accumulation in the BM of patients with T2D and also show deleterious consequences of the ADPC secretome on the BM microenvironment

T2D BM-MSCs are primed to an adipogenic fate

Patients with T2D have a twofold accumulation of BM-ADPCs, which is caused by both hypertrophy and hyperplasia of the BMAT. Adipocyte hypertrophy occurs before hyperplasia (34), which suggests that T2D subjects' BMAT has reached "peak storage" and that more ADPCs need to differentiate from the BM-MSC pool to meet the energy storage demands of the BM. We show that BM-MSCs of T2D patients have an increased intrinsic adipogenic potential, without changing their osteogenic potential. Undifferentiated BM-MSCs of T2D have increased expression of pro-adipogenic factors and decreased expression of anti-adipogenic factors at steady state. C/EBPα is central for ADPC differentiation and lipid accumulation (35). Hence, high levels of C/EBPα suppose a "priming" of BM-MSC towards adipogenesis. PDGFRβ, another important regulator of BM-MSC fate determination, is negatively associated with osteogenesis (36), while being involved in diabetic BM adipogenesis, as supported by lineage tracing experiments in mice showing PDGFRβ-positive mural stem cells are the originators of adipose tissue hyperplasia under high-fat diet conditions (37). Our finding of a twofold increase in PDGFRβ-positive BM-MSCs of T2D patients further supports a readiness towards fat accumulation.

Adipogenesis feedback loop between BMAT and BM-MSCs in the diabetic BM

We show that the secretome of the BMAT can change the BM-MSC fate commitment *in vitro*. The CM of cultured BM-ADPCs from ND subjects potentiates BM-MSC osteoblast differentiation, which goes counter with the established paradigm. But under T2D conditions, we found that osteoblastogenesis is inhibited by diabetic BM-ADPC secreted factors. This suggests that it is not the BMAT volume that negatively modulates osteogenesis, but the pathophysiological status of the adipose tissue itself. We also demonstrate that the secretome of T2D-BM-ADPCs enhances the adipogenic activity of BM-MSCs.

This phenomenon creates a vicious cycle of fat accumulation in the diabetic BM, as the appearance of mature ADPCs will send more adipogenic signals to the BM-MSCs, which will differentiate into more ADPCs, the final result consisting of BM-MSC depletion, lowered osteogenesis, and aberrant adipogenesis.

T2D alters adipokine expression and secretion in BM-ADPCs

Analysis of ADPC transcripts and secretome showed the upregulation of LEP and RETN and downregulation of ADIPOQ in human BM-ADPCs, which follows the patterns observed in other white adipose deposits of T2D patients (38). LEP has been shown to induce adipogenesis and inhibit osteoblastogenesis in the BM (39). RETN is also interesting in regard to bone remodelling as it activates osteoclast differentiation in the BM (40). The decrease in ADIPOQ production is of particular relevance because BMAT produces more ADIPOQ than the other WAT depots (13). This would suggest that the decrease in circulating levels of ADIPOQ in T2D patients (41) is dependent on BMAT production of this adipokine. MMP2 is another factor which could contribute to the positive feedback loop of adipogenesis in the BM of T2D individuals, possibly through a previously reported *in-vitro* action on pre-ADPC differentiation (42).

T2D-BM-ADPCs stimulate adipogenesis in BM-MSCs *via* MCP-1

We decided to conduct a more in-depth investigation on MCP-1 for several reasons. Firstly, MCP-1 showed the highest amplitude of increase at the mRNA and the secreted protein levels in T2D-BM-ADPCs. Secondly, it is known that high levels of circulating MCP-1 are associated with insulin resistance (19) and that MCP-1 is involved in osteoclastogenesis, bone reabsorption (43) and pre-ADPC differentiation *via* the transcription factor MCPIP (24, 25). And thirdly, we found that this increase is conserved among species as MCP-1 levels are augmented in the BM-ADPCs of Db/Db T2D mice. No previous study has examined the possibility that BM-ADPC-produced MCP-1 drives the conversion of MSCs into new ADPCs in diabetes. We verified this possibility by pharmacological antagonism of the MCP-1 receptor on MSCs that were exposed to the BM-ADPC CM through an adipogenic induction assay. Results indicate that RS504393 inhibits the transcription of the adipogenic markers ADIPOQ,

PPARγ and FABP4, resulting in the complete abolition of T2D-ADPC ability to induce MSCs into making new ORO positive ADPCs.

Systemic inhibition of MCP-1 rescues BM adipogenic remodelling and increases osteocyte density in T2D bones

In-vivo treatment of Db/Db T2D mice with RS504393 for 8 weeks resulted in the amelioration of several pathological features. Similar to the study published by Kang et al. (21), we observed a slower weight gain in treated Db/Db mice, which was reflected by a lowered epididymal fat pad weight through reduction in the size of ADPCs. RS504393 treatment also lowered urine glucose and plasma Hba1c levels, indicating that MCP-1 receptor antagonism may benefit metabolic control.

More importantly, we show that MCP-1 antagonism decreases the accumulation of fat in the BM of diabetic mice. BMAT reduction by RS504393 was mainly due a lowered number of ADPCs associated with a shift towards smaller ADPCs. This suggests that MCP-1 antagonism not only limits adipogenesis but also promotes the formation of more "healthy" metabolically active ADPCs (44).

In Db/Db mice, the bone thickness was half as thin as observed in control ND Wt/Db mice and RS504393 did not rescue this phenotype. However, we did observe changes in bone-regulating cell populations. In untreated Db/Db mice, the density of osteocytes was greatly reduced as compared with the ND Wt/Db mice. Osteocytes are responsible for bone mechano-sensing, homeostasis and remodelling as they coordinate activity of osteoclasts and osteoblasts (45). It has been shown that a reduction of osteocyte lacunae in older patients is associated with defects in bone remodelling and fracture healing (46, 47). MCP-1 antagonist treatment of Db/Db mice increased osteocyte density in the femoral bone to an intermediate level between ND Wt/Db and untreated Db/Db. Concurrently, RS504393 treatment reduced the levels of endosteal osteoclasts to ND levels. Taken together, these results indicate that MCP-1 antagonism can ameliorate bone health in T2D.

MCP-1 inhibition rescues the deficit in long-term HSCs in T2D BM

We showed a decrease in total HSC levels in the two T2D groups treated with the MCP-1 antagonist or its vehicle as compared with ND. This data confirms the depletive action of diabetes and also suggests that MCP-1 does not directly contribute to HSC rarefaction. However, when looking at the

abundance of specific sub-populations, we found that MCP-1 antagonism rescued the T2D-associated primitive LT-HSC deficit restoring the levels observed in ND mice. *In-vitro* studies indicate that the effect of MCP-1 antagonism on HSC phenotypes is indirect and may depend upon a better metabolic control and the removal of the inhibitory action of BMAT on hematopoiesis. Reduced adipocyte presence in the BM signifies a decrease in secretion of factors that negatively affect HSC maintenance, thus permitting the recovery of proper primitive LT-HSC levels. In line with this, BM reconstitution studies showed that inhibition of adipogenesis is beneficial for hematopoietic tissue recovery (33). Altogether, these findings suggest that secreted factors other than MCP-1 are responsible for the paracrine influence of T2D-BM-ADPCs on HSC lineage progression and that treatment with RS504393 corrects the proportion of LT- and SC-HSCs indirectly through amelioration of general and local conditions, such as improved metabolic control and inhibition of BM adipogenesis.

In conclusion, this study highlights a key paracrine mechanism by which adipose tissue accumulates in the diabetic BM and perpetuates BM-MSC adipogenic differentiation. This study demonstrates for the first time that MCP-1 inhibition provides a means for global protection of the BM microenvironment in diabetes, reducing BMAT volume and ADPC size and supporting bone health and hematopoiesis. These results have important clinical and therapeutic implications for the maintenance of the BM and bone integrity. We suggest that clinical MCP-1 receptor antagonism could pave the way to novel treatments of T2D and associated co-morbidities.

Author Contribution

Dr David Ferland-McCollough contributed to the article by establishing the hypothesis and research protocol of this study, generating the data of figures 2 through 8, as well as drafting and correcting the manuscript. Dr Davide Maselli contributed to the article by generating the data of figure 1, correcting the manuscript and participating in the scientific discussion on the structure and data of the paper. Dr Gaia Spinetti and Maria Sambataro contributed to the article by providing human bone marrow histology samples, correcting the manuscript and participating in the scientific discussion on the structure and data of the paper. Professor Ashley Blom and Mr Niall Sullivan contributed to the

article through their surgical expertise and their collection of human bone marrow. Professor Madeddu is the principal investigator of the research project and contributed to the article by obtaining BHF funding, generating ideas, participating in the scientific discussion on the structure and data of the paper and participating in the drafting and correction of the article as well as providing ivaluable mentoring to the first author Dr David Ferland-McCollough.

The two corresponding authors; Dr David Ferland-McCollough and Professor Paolo Madeddu are the guarantors of this study.

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Figure legends

Figure 1. Adipocyte accumulation in bone marrow of type 2 diabetic patients compared to non-diabetic controls.

Haematoxylin & Eosin staining of human femoral bone samples from non-diabetic (ND) and type 2 diabetic (T2D) subjects (a). Quantification of % of total bone marrow (BM) area occupied by adipocytes (ADPCs) (b), density (c), size (d) and frequency of ADPCs according to attribution to size categories (e). Values are means with standard error bar. *P<0.05, **P<0.01, and ***P<0.001 vs. ND. N=8 biological replicates per group.

Figure 2. Bone marrow mesenchymal stromal cells from type 2 diabetic patients have an increased intrinsic potential to differentiate into the adipocyte lineage.

Basal mRNA expression levels of adipogenesis regulators in bone marrow mesenchymal stromal cells (BM-MSCs) from non-diabetic (ND) and type 2 diabetic (T2D) subjects (a). Flow Cytometry analysis of BM-MSCs (b) consisted of gating live single cells, selecting for dual-positive CD105-CD73 MSCs FMO (i), and finally examining the frequency of cell population fractions positive for PDGFRβ (ii) and PREF1 (iii). Bar graphs showing the quantification of $CD73^{pos}CD105^{pos}$ (iv), $CD73^{pos}CD105^{pos}PDGFR\beta^{pos}$ (v), and $CD73^{pos}CD105^{pos}PREF1^{pos}$ (vi) populations. Adipocyte (ADPC) differentiation of BM-MSCs (c). ADPCs are stained with Oil Red (ORO positive cells) (i) and quantified by morphometry of microscopic images (ii). qPCR analysis of *FABP4* (iii), *ADIPOQ* (iv) and *PPARγ* (v) mRNA expression in BM-MSCs at different times of the adipogenesis induction assay. Values are means with standard error bars. *P<0.05, **P<0.01, and ***P<0.001 vs. ND, $^{+}$P<0.05 and $^{+++}$ P<0.001 vs. time 0 of the adipogenic assay. N=4 biological replicates per group.

Figure 3. Secreted factors from human mature bone marrow adipocytes paracrinally regulate the differentiation of bone marrow mesenchymal stromal cells into adipocyte and osteoblasts.

Adipocyte (ADPC) differentiation of bone marrow (BM) mesenchymal stromal cells (BM-MSCs) from non-diabetic (ND) subjects following treatment with regular induction medium alone (No CM), or the same medium supplemented with ND-BM-ADPC conditioned medium (ND CM), or type 2 diabetic (T2D) BM ADPC conditioned medium (T2D CM) (a). ADPCs stained with Oil Red O pointed by arrows (i) and related quantification (ii). Values are means with standard error bars. ***P<0.001 vs. No CM and $^{+++}$P<0.001 vs. ND CM. N=7 biological replicates per group. qPCR analysis of *ADIPOQ* (iii), *PPARγ* (iv) and *FABP4* (v) mRNA expression at different times of the adipogenesis induction assay. Values are

means with standard error bars. *P<0.05 and ***P<0.001 vs. No CM, $^{+}$P<0.05 and $^{+++}$P<0.001 vs. ND CM. N=4 biological replicates per group. Osteoblast differentiation of BM-MSCs treaded with No CM, ND CM, or T2D CM (b) Alkaline-phosphatase positive osteoblasts (i) and related quantification (ii). qPCR analysis of *RUNX2* (iii) mRNA expression after 2 weeks of induction. Values are means with standard error bars. *P<0.05 and **P<0.01 vs. No CM, $^{+}$P<0.05 and $^{+++}$P<0.001. N=4 biological replicates per group.

Figure 4. Type 2 diabetes modulates the expression and secretion of adipokines and cytokines in bone marrow adipocytes.

mRNA expression analysis by qPCR (a) of human mature bone marrow adipocytes (BM-ADPCs) from non-diabetic (ND) and type 2 diabetic (T2D) subjects (a). Bar graphs showing levels of adipokines (i) and cytokines (ii). ELISA quantification of secreted factors in conditioned media (CM) from BM-ADPCs of the two groups (b). Secreted factors measured are Leptin (i), Resistin (ii), Adiponectin (iii), PEDF (iv), MCP-1 (v), MMP2 (vi), IFNγ (vii), TNFα (viii) and Angiopoietin1 (ix). Values are means with standard error bars. *P<0.05, ** P<0.01, and ***P<0.001 vs. ND. N=6 biological replicates per group.

Figure 5. Antagonism of MCP-1 signalling by a CCR2 antagonist inhibits the promotion of adipogenesis in non-diabetic bone marrow mesenchymal stromal cells exposed to the conditioned medium from type 2 diabetic bone marrow adipocytes.

Bone marrow mesenchymal stromal cells (BM-MSCs) from non-diabetic subjects (ND) underwent the adipogenic assay in the presence or absence of the specific MCP-1 receptor antagonist RS504393. Three groups were studied where BM-MSCs were incubated with regular induction medium alone (No CM), or the same medium supplemented with ND-BM-ADPC conditioned medium (ND CM), or type 2 diabetic (T2D) BM ADPC conditioned medium (T2D CM). ADPCs stained with Oil Red O pointed by arrows (a) and related quantification (b). Using the same protocol, we performed qPCR analyses of *ADIPOQ* (c), *PPARγ* (d), *FABP4* (e), and *MCPIP* (f) mRNA at the end of the adipogenesis induction. Values are means with standard error bars. *P<0.05, **P<0.01, and ***P<0.001 vs. No CM, $^{\#}$P<0.05 and

##P<0.01 vs. ND CM, &P<0.05,&&P<0.01, and &&&P<0.001 vs. No inhibitor. N=5 biological replicates per group.

Figure 6. *In-Vivo* Inhibition of MCP-1 signalling improves metabolic control in T2D diabetic mice.

An *in-vivo* study was performed in mice to assess the effect of MCP-1 antagonism on systemic and bone marrow (BM) endpoints. This figure illustrates the systemic outcomes. Body weight (BW) progression and fat deposit profiles of non-diabetic (ND) vehicle (DMSO)-treated Wt/Db mice (ND WT DMSO group), DMSO-treated obese type 2 diabetic (T2D) Db/Db mice (T2D DD DMSO group), and MCP-1 inhibitor (RS504393)-treated T2D Db/Db mice (T2D DR RS504393 group) (a). Weight increase of mice during the 8 weeks of treatment (i). Pericardial, inguinal, and epididymal fat pad weight (ii). Histology of epididymal fat pad (iii) and size quantification of adipocytes (ADPCs) of the epididymal fat pad (iv). Glucose and glycation profiles of mice in the different treatment groups (b). Urine glucose levels measured weekly for 8 weeks with urinalysis sticks (i) and glycated hemoglobin (HbA1c) plasma levels at week 8 of treatment (ii). Values are means with standard error bars. *P<0.05, **P<0.01, and ***P<0.001 vs. ND WT, #P<0.05 and ### P<0.001 vs. T2D DD group. N=6 biological replicates per group.

Figure 7. *In-Vivo* inhibition of MCP-1 signalling decreases fat accumulation and increases osteocytes content in bone marrow of T2D diabetic mice.

An *in-vivo* study was performed in mice to assess the effect of MCP-1 antagonism on systemic and BM endpoints. This figure illustrates the BM outcomes with regard to fat and bone. Abbreviations are the same as in Figure 6. Histology of mouse femoral BM (a). Haematoxylin & Eosin staining image at 4x and 20x magnification (i), quantification of ADPC coverage over total BM area (ii), average ADPC size quantification (iii), ADPC size group distribution (iv), and quantification of the number of ADPCs per microscopic field (v). Analyses performed on the BM whole cell population from tibia at week 8 (b) with mRNA quantification of murine *Mcp-1* (i) and *Mcpip* (ii) expression. MPC-1 levels measured by ELISA at week 8 of treatment in plasma (iii) and cell-free BM lavage (iv). Histology of mouse femoral bone (c). Haematoxylin & Eosin staining of the femoral bone and osteocytes pointed by arrows (i) with

bone thickness measurement (ii), osteocyte density (iii) and osteoclast density (iii). Values are means with standard error bars. *P<0.05, **P<0.01 and ***P<0.001 vs. ND WT, $^{\#}$P<0.05 and $^{\#\#\#}$ P<0.001 vs. T2D DD group. N=6 biological replicates per group.

Figure 8. *In-Vivo* inhibition of MCP-1 signalling rescues the content of primitive hematopoietic stem cells in bone marrow of T2D diabetic mice.

An *in-vivo* study was performed in mice to assess the effect of MCP-1 antagonism on systemic and BM endpoints. This figure illustrates the BM outcomes with regard of macrophages (M) and hematopoietic stem cells (HSCs). Abbreviations are the same as in Figure 6. Flow cytometry analysis of tibia BM. Analysis of the bone marrow HSC population (a) were total BM HSCs were defined as $Lin^{neg}cKit^{pos}Sca\text{-}1^{pos}$, LT-HSCs as $Lin^{neg}cKit^{pos}Sca\text{-}1^{pos}CD34^{neg}$, and ST-HSCs as $Lin^{neg}cKit^{pos}Sca\text{-}1^{pos}CD34^{pos}$ (i). Quantification of HSCs (ii), LT-HSCs (iii), and ST-HSCs (iv) in total BM cells. Values are means with standard error bars. *P<0.05, **P<0.01 and ***P<0.001 vs. ND WT, $^{\#}$P<0.05 vs. T2D DD group. N=6 biological replicates per group. In a separate *in-vitro* experiment, murine BM mononuclear cells were exposed to BM-ADPC CM from ND or T2D subjects (using unconditioned medium as control) for 96 hours in the presence or absence of the MCP-1 antagonist and the HSC population was analyzed by flow cytometry (b). Flow cytometry gating (i). Quantification of LT-HSCs (ii) and ST-HSCs (iii). Values are means with standard error bars. *P<0.05 and **P<0.01 vs. corresponding No CM group. N=4 biological replicates per group.

Supplementary Online Information

SOI Table 1. Patients Characteristics and Use in Experiments

Table illustrating anonymized patient identification, gender, age, Body Mass Index (BMI) and presence and duration of type 2 diabetes. “Not reported” refer to particular cases we access to this data from clinical records was not allowed.

SOI Table 2. List of DNA Primers Used for qPCR Experiments

SOI Table 3. List of Antibodies Used in Flow Cytometry Experiments

SOI Figure 1: Levels of MCP-1 protein and *Mcp-1* mRNA levels in BMAT adipocytes from non-diabetic Wt/Db and T2D Db/Db mice. Values are means with standard error bars. *P<0.05 and Wt/Db. N=4 biological replicates per group.

SOI Figure 2: Body weight growth and cumulative food intake in mice undergoing the in vivo experiment with MCP-1 antagonism.

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